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Detection of Coxsackie B Virus RNA Sequences in Whole Blood Samples From Adult Patients at the Onset of Type I Diabetes Mellitus

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Enteroviruses may be linked to insulin-dependent diabetes mellitus (IDDM). The prevalence of enteroviral (EV) infection at onset of adult IDDM was investigated by detection of specific EV sequences in peripheral blood using a reverse transcription and a seminested polymerase chain reaction (seminested RT-PCR). EDTA-treated whole blood samples taken from 12 newly diagnosed IDDM patients with ketosis or ketoacidosis were examined. The comparison groups were 12 adult patients suffering from metabolic decompensation in the course of IDDM, 12 adult patients with decompensated non-IDDM, and 15 healthy adults without any presumed EV infection or metabolic disease. EV genome was detected in five of 12 (42%) newly diagnosed IDDM patients and in one of 12 (8%) patients in the course of IDDM. By contrast, none of the 12 non-IDDM patients and none of the 15 healthy adults had EV sequences in whole blood. Subsequent sequencing of the EV PCR products from the six positive patients showed a significant homology with Coxsackie B3 or B4 viruses, and some common patterns were observed among the sequences. The present study demonstrates that Coxsackie B virus RNA sequences can be detected in peripheral blood from patients at the onset or in the course of IDDM and provides evidence for a role for enteroviruses in adult type I diabetes. ***J. Med. Virol. 52:121–127, 1997.***

KEY WORDS: enterovirus infection; insulin-dependent diabetes mellitus; seminested RT-PCR; genetic typing of viral strains

INTRODUCTION

Type I insulin-dependent diabetes mellitus (IDDM) is characterised by infiltration of lymphocytes into the islets of Langherans and irreversible destruction of pancreatic insulin-secreting β cells. Although genetic markers for susceptibility to IDDM have been clearly established, recent studies, including those on discordance for IDDM between identical twins and environmentally produced diabetes in animals, have suggested the role of environmental factors in the expression of the disease [Barnett et al., 1981; Laporte et al., 1987].

Environmental agents such as viruses have been identified as potentially important determinants of IDDM [Yoon, 1991]. The association between Coxsackie viruses of group B and pancreatic diseases including diabetes has been recognised for many years [Grist et al., 1978; King et al., 1983]. Experimental studies have clearly demonstrated that Coxsackie viruses B4 could induce pancreatitis, insulitis, β-cell necrosis, and diabetes in animals [Szopa et al., 1990, 1992; Kang et al., 1994; Rewers and Atkinson, 1995]. On one occasion, a Coxsackie virus B4 strain was isolated from the pancreas of a child who died at onset of IDDM, and the isolate was diabetogenic for mice [Yoon et al., 1979]. However, evidence for enteroviral (EV) involvement in human IDDM was based largely on the detection of specific anti-Coxsackie viruses B immunoglobulin M (IgM) as well as on the action of neutralising antibodies in the sera of children at the onset of the disease [Banatvala et al., 1985; Helfand et al., 1995; Rewers and Atkinson 1995]. These antibody detection techniques are limited in their methodology and interpretation and, therefore, furnished controversial data on the role of EV infection in IDDM [Rewers and Atkinson 1995].

Recently, new EV genomic sequences and genetic data have allowed the development of specific EV genomic detection techniques [Rotbart, 1991]. Using reverse transcription-polymerase chain reaction (RT-

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PCR), amplification of highly conserved EV sequences has made it possible to detect a broad array of EV serotypes with a high degree of specificity and sensitivity in biological samples [Rotbart, 1990]. By using this genomic amplification strategy, the presence of EV RNA has been shown lately in nine of 14 sera taken from children at the onset of IDDM, whereas only two of 45 appropriate control samples were positive, suggesting a role for enteroviruses in childhood type I diabetes [Clements et al., 1995].

In the present study, the prevalence of EV infections at the onset of IDDM was investigated in adult patients using reverse transcription and seminested PCR. The detection of EV RNA was carried out in whole blood samples from adult patients suffering from newly diagnosed IDDM vs. adult patients with metabolic ketosis decompensation in the course of IDDM vs. NIDDM adult patients suffering from metabolic decompensation vs. healthy adult subjects without any suspected EV infection and metabolic disease. In addition, we performed a genetic typing of amplified EV RNA sequences by direct sequencing of cDNA amplicons and subsequent computer data analysis.

MATERIALS AND METHODS

Patient Group

The 12 newly diagnosed IDDM patients in the study were adults referred by a general practitioner from the Emergency Department to the Department of Endocrinology and Metabolic Diseases of the Lille Centre Hospital, where they were examined by an endocrinologist and a diagnosis of metabolic decompensation was established. From September 1995 to March 1996, six males and six females admitted to the hospital with ketosis, including seven patients with diabetic ketoacidosis (DKA) (age 28.2 ± 10.4 years), were studied. For each patient, a venous whole blood sample in EDTA was taken at time of diagnosis of metabolic disease. All blood samples were rapidly sent to the virology laboratory, where they were aliquoted in 100 μl of blood sample, mixed with RNase inhibitor (20 IU per 100 μl) (Boehringer, Mannheim, Germany) and stored at −80°C until use.

Control Groups

A first control group included 12 adults (seven males and five females) with metabolic ketosis decompensation in the course of IDDM (previously diagnosed IDDM patients), including seven with DKA (age 32.6 ± 13.3 years). In a second control group, we included 12 patients (six males and six females) with metabolic decompensation in the course of NIDDM (age 57.9 ± 13.3 years). All of these NIDDM patients had been diagnosed previously and treated in the Department of Endocrinology of the Lille Centre Hospital. A third control group was made of 15 healthy subjects (six males and nine females age 28.2 ± 10.4 years). These healthy subjects were selected by biological and clinical assessments and those suffering from any suspected infectious or metabolic disease were excluded from this study. A venous whole blood sample was taken at the time of metabolic decompensation for IDDM and NIDDM patients and at the time of inclusion for healthy subjects. All clinical samples were sent to the virology laboratory and treated as described above.

Virus and Cells

Cell culture fluids of Coxsackie virus B 1–6, poliovirus 1–3 reference strains (ATCC collection, Rockville, MD), were propagated in Hep2 cells (Whittaker Bioproducts, Walkersville, MD). Echovirus 11, cytomegalovirus (CMV), herpes simplex viruses 1 and 2 (HSV 1 and 2), and varicella zoster virus (VZV) were isolated from clinical specimens and serotyped in our laboratory [Andreoletti et al., 1996]. For each viral suspension, a microtitration plate methodology was performed and the results were expressed as plaque-forming units (pfu) of virus per millilitre of cell culture fluid.

RNA Extraction

Native RNA was extracted with RNAplus (Bioprobe Systems, Montreuil Sous Bois, France) from clinical specimens [Chomczynski and Sacchi, 1987]. One hundred microlitres of whole blood were homogenised in 1 ml of RNAplus (guanidinium/phenol) and 200 μl of chloroform. Extracted RNA was then precipitated with isopropanol (v/v) at 4°C for 20 min. After centrifugation (12,000 *g* for 15 min), the pellet was washed twice in 75% ethanol and vacuum-dried. The RNA was then dissolved in 50 μl of diethylpyrocarbonate (DEPC)-treated water and used in the RT-PCR assays.

RT-PCR Procedure

Oligonucleotides. All oligonucleotides used for reverse transcription, first PCR, and second-round PCR were located within the 5′NC region, which is highly conserved among the EV serotypes. The external primers (EV1, 5′-CAAGCACTTCTGTTTCCCCGG-3′; EV2, 5′-ATTGTCACCATAAGCAGCCA-3′) generate a 435 base pair (bp) fragment, whereas the use of the primer EV1 and internal primer EV3 (5′-CTTGCGCGTTACGAC-3′) generates a 362 bp PCR product. These primers were previously described and used by Leparc et al. [1994].

Reverse transcription. RT-PCR was carried out as described previously [Andreoletti et al., 1996]. Briefly, complementary desoxyribonucleic acid (cDNA) was synthesised in a total volume of 12.5 μl containing 2.5 μl of extracted RNA; 12.5 pmol of primer EV2; 20 IU reverse transcriptase; 20 IU RNAsin; 0.15 μl of a solution containing 25 mM of dATP, dCTP, dGTP, and dTTP (Boehringer); 2.5 μl RT buffer 10×; and 3.80 μl of sterile water. The reaction was carried out at 37°C for 60 min and stopped by heating the samples for 5 min at 95°C.

PCR amplification. Then, 37.5 μl of PCR reaction mixture consisting of 5 μl of 10× reaction buffer, 1.25 IU Taq-DNA polymerase, 12.5 pmol of EV1 primer, 0.3 μl of the previously described dNTPs solution, and 29.1 μl of sterile water were added directly to the cDNA sample. DNA amplification was carried out

in an MJ Research thermocycler PTC 200 (MJ Research, Watertown, MA) for 35 cycles (denaturation 95°C, 30 sec; annealing 50°C, 45 sec; extension 72°C, 1 min).

Seminested amplification. A second run PCR was carried out as described previously by Leparc et al. [1994]. Briefly, 1 μl of the amplified products was added to 49 μl of the previously described PCR mixture, including 12.5 pmol of primers EV1 and EV3. Amplification was undertaken as described above.

PCR controls. In order to verify the absence of contamination, each PCR analysis included a positive control consisting of 0.5 μg of RNA extracted from 10^4 pfu of poliovirus 3 (Sabin) and two negative controls of retrotranscription and amplification consisting of 2.5 μl of sterile water, which were added in place of the test sample [Rotbart, 1990]. In order to avoid contamination, each step of the RT-PCR procedure was undertaken in a separate room, the reagents were aliquoted and stored at −20°C, specific pipettes were used for each step, and work surfaces were treated with ultraviolet light for 30 min [Kwok and Higuchi, 1989].

Detection of PCR products. An aliquot of amplified RT-PCR product (15 μl) was subjected to electrophoresis under 100 V in a 2% agarose gel containing 0.5 μl/ml of ethidium bromide (Sigma, St. Louis, MO), and a 100 bp DNA ladder (GIBCO BRL, Paris, France) was used as molecular weight marker. DNA bands were observed under ultraviolet illumination and photographed with Polaroid MP4 high-speed film (Bioblock Scientific, Illkirch, France).

Sequence Analysis of Amplified Products

The nucleotide sequence of fragments was determined by double strand DNA cycle sequencing using a Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Electrophoresis and analysis of DNA sequence reactions were carried out using an automated DNA sequencer (model 373 A; Applied Biosystems, Roissy Charles de Gaulle, France). The sequencing primers were those used in the second run of PCR amplification. Prior to sequencing, the PCR products were purified using a Qiaquick gel extraction kit (Qiagen, Hilden, Germany). Both strands of the DNA fragments were sequenced. The sequence data were then entered into a Macintosh computer and analysed automatically with the Usedit and Sequence Navigator programmes (Applied Biosystems). These multiple sequence alignment programmes were used for genetic typing of amplified sequences. Previously published EV sequences used in the comparisons were obtained from the Genbank data base [Clements et al., 1995].

RESULTS

Validity of EV RNA Detection Assay

The sensitivity of the EV-RNA detection assay was assessed by limit detection of the signal in serial tenfold dilutions of Coxsackie virus B3 (Nancy strain) added to sterile human EDTA whole blood. Our seminested PCR procedure was able to detect ten RNA copies of CVB3 per 100 μl of human whole blood according to the protocol described by Severini et al. [1993] (data not shown). The specificity of the seminested RT-PCR assay was confirmed by the presence of amplification products with RNA extracted from polioviruses 1–3, Coxsackie viruses B1–6, and echovirus 11 and by the absence of amplified cDNA with RNA extracted from herpes virus family-infected and noninfected Hep2 cells [Andreoletti et al., 1996] (data not shown).

EV RNA Detection in Whole Blood Specimens

Within the healthy control group, none of the 15 specimens taken from adult subjects without any suspected infectious or metabolic disease was positive by EV RNA detection assay (Table I). Within the group of control patients suffering from metabolic decompensation in the course of NIDDM (previously diagnosed NIDDM), EV RT-PCR was negative in the 12 samples tested. In order to assess whether EV-specific RNA sequences could be detected in patients suffering from metabolic decompensation in the course of IDDM, we examined 12 whole blood samples taken from previously diagnosed IDDM patients suffering from metabolic ketosis decompensation. Within this control group, only one of 12 (8%) patients (a female) was positive for EV RNA and was suffering from DKA (Table I; Fig. 1, lane 11).

In the group of newly diagnosed IDDM patients, EV nucleic acid sequences were found in five (42%) of 12 patients ($P < 0.01$ vs. healthy controls, χ^2 Fischer exact test). Figure 1 shows examples of detection by ethidium bromide-stained agarose gel after seminested RT-PCR. Among the five EV-positive patients of the newly diagnosed IDDM group (three males and two females), four were suffering from DKA and one from metabolic ketosis decompensation without acidosis (Table I). However, the statistical analysis of the RT-PCR data demonstrated that there was no significant difference in the prevalence of EV infection between newly diagnosed IDDM (42%) and previously diagnosed IDDM (8%) patients with metabolic decompensation ($P = 0.0775$, χ^2 Fischer exact test) (Table I). Moreover, for each IDDM patient, human lymphocyte antigen (HLA) class II typing was carried out according to the procedure described by Danzé et al. [1995]. The results showed that only two of the six EV-positive patients had an HLA-DR haplotypic combination conferring a moderate or high risk for IDDM, suggesting an aetiological role of enteroviruses in the pathogenesis of IDDM (Table II).

Sequence Analysis of EV-Positive Samples

In order to carry out genetic typing of the six EV-positive samples, the cDNA amplicons were sequenced in both directions and the data were then compared to the Coxsackie virus B3 (CVB3), B4 (CVB4), and B4 diabetogenic in mice (CVB4D) sequences [Clements et al., 1995].

Sequence data from four PCR amplicons from the newly diagnosed IDDM patients demonstrated a high

TABLE I. Detection of Enteroviral RNA in Whole Blood Samples Taken From Healthy Subjects and From Adult Patients Suffering From Metabolic Decompensation at the Onset of IDDM, in the Course of IDDM, and in the Course of NIDDM

Groups	Total (n)	RT-PCR Positive	RT-PCR Negative	Percentage positive
Newly diagnosed IDDM[a]*	12	5	7	42
Previously diagnosed IDDM[b]	12	1	11	8
Previously diagnosed NIDDM[c]	12	0	12	0
Healthy adult controls[d]	15	0	15	0

[a]Newly diagnosed IDDM, group of patients suffering from diabetic ketoacidosis or diabetic ketosis at the onset of insulin dependent diabetes mellitus.
[b]Previously diagnosed IDDM, group of patients suffering from diabetic ketoacidosis or diabetic ketosis in the course of insulin dependent diabetes mellitus.
[c]Previously diagnosed NIDDM, group of patients suffering from diabetic ketosis in the course of non-insulin-dependent diabetes mellitus.
[d]Healthy adult controls, group of adult subjects without any suspected enteroviral infection or metabolic disease.
*$P < 0.01$ vs. healthy controls; $P = 0.0775$ vs. previously diagnosed IDDM (χ^2 Fischer exact test).

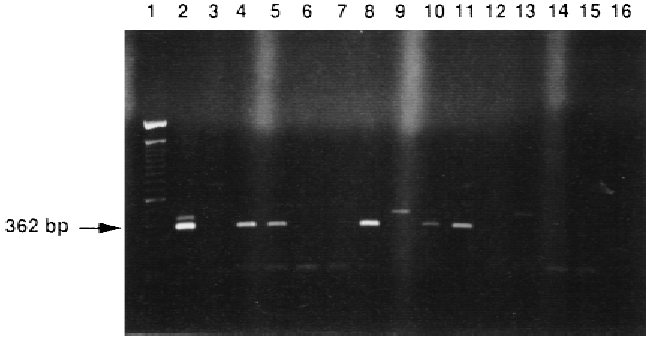

Fig. 1. Agarose gel electrophoresis revelation of seminested RT-PCR products. This amplification was carried out in whole blood from adult patients suffering from metabolic decompensation at the onset of IDDM (lanes 8–10), in the course of IDDM (lanes 11–14), and in the course of NIDDM (lanes 15 and 16). An enterovirus-specific fragment of 362 bp can be seen in lanes 4, 5, 8, 10, and 11 but not in lanes 3, 6, 7, 9, and 12–16. Lane 2 corresponds to the positive control and lane 3 to the negative control; lane 1, molecular weight markers (100 bp DNA ladder).

degree (97–99%) of similarity with CVB3 and a lower percentage of sequence homology with CVB4 and CVB4D (85.5–87.4% and 87.3–88.4%, respectively) over 320 nucleotides from 189 to 509 at the 5′ untranslated region (Fig. 2, AD4–7). Sequence data from one of the PCR amplicons obtained from the newly diagnosed IDDM group showed a significant degree of homology with CVB4 (99%) over the same nucleotidic region (Fig. 2, AD10). The sequence analysis of the PCR amplicon obtained from the positive patient from the previously diagnosed IDDM group showed a significant degree of similarity with CVB4 (98.7%) and CVB4D (91.5%) (Fig. 2, AD11).

Moreover, these EV sequences obtained in the cases of adult IDDM have several features in common. At position 234, five of the six diabetic sequences have thymidine, unlike CVB3, CVB4, and CVB4D (cytosine). At position 229, the two sequences showing a higher percentage of homology with CVB4 (AD10 and 11) have adenine, unlike CVB4 and CVB4D (cytosine). However, without further sequence data from a wider range of viral amplicons from IDDM adult patients, it is not possible to evaluate the significance of these nucleotidic mutations.

DISCUSSION

The isolation of a CVB4 from the pancreatic tissue of a child with DKA indicated that EV infection could be either responsible for or a triggering factor of IDDM [Yoon, 1979]. Further support for this concept came from seroepidemiological studies which reported an association between IDDM and antibodies to enteroviruses. However, the data based on Coxsackie virus serology at the onset of IDDM, although suggestive, were not convincing as evidence for the involvement of enterovirus [Helfand et al., 1995; Rewers and Atkinson, 1995].

EV nucleic acids can now be detected reliably in various body tissues to evaluate whether infection is present [Rotbart, 1990]. To study the prevalence of EV RNA detection in peripheral blood from adult patients at the onset of IDDM, we selected controls divided into three groups: one group included adult patients with metabolic ketosis in the course of IDDM, the second included NIDDM patients with metabolic decompensation, and the third included healthy adult subjects without any suspected infectious or metabolic pathology. Since human peripheral blood mononuclear cells (PBMC) can harbour enteroviruses during and after a viremic phase, we extracted the native RNA from whole blood samples [Vuorinen et al., 1994]. Then, we used a seminested RT-PCR strategy allowing a rapid and sensitive detection of a wide range of EV serotypes in biological samples [Leparc et al., 1994].

In the control group of NIDDM patients, the fact that none of the whole blood samples was EV RNA-positive is an argument in favour of EV involvement as the primary aetiological agent in metabolic decompensation during type II diabetes. Furthermore, none of the samples from the healthy control group was positive for EV detection, thus demonstrating the absence of persistent infection in whole blood from immunocompetent patients.

TABLE II. HLA-DR Typing in IDDM Patients Positive by Enteroviral RT-PCR

Patient	Sex	Age (years)	Sporadic case (Yes/No)	HLA genotype DR (risk to develop IDDM)	Enterovirus genotype
Newly diagnosed IDDM patients					
1	F	28	Y	DR1/DR4 (low risk)	CVB3
2	M	29	Y	DR7/DR5 (no risk)	CVB3
3	F	14	Y	DR4/DR3 (high risk)	CVB3
4	M	28	Y	DR1/DR3 (low risk)	CVB3
5	M	53	Y	DR7/DR3 (moderate risk)	CVB4
Previously diagnosed IDDM patient					
1	F	24	Y	DR4/DR4 (low risk)	CVB4

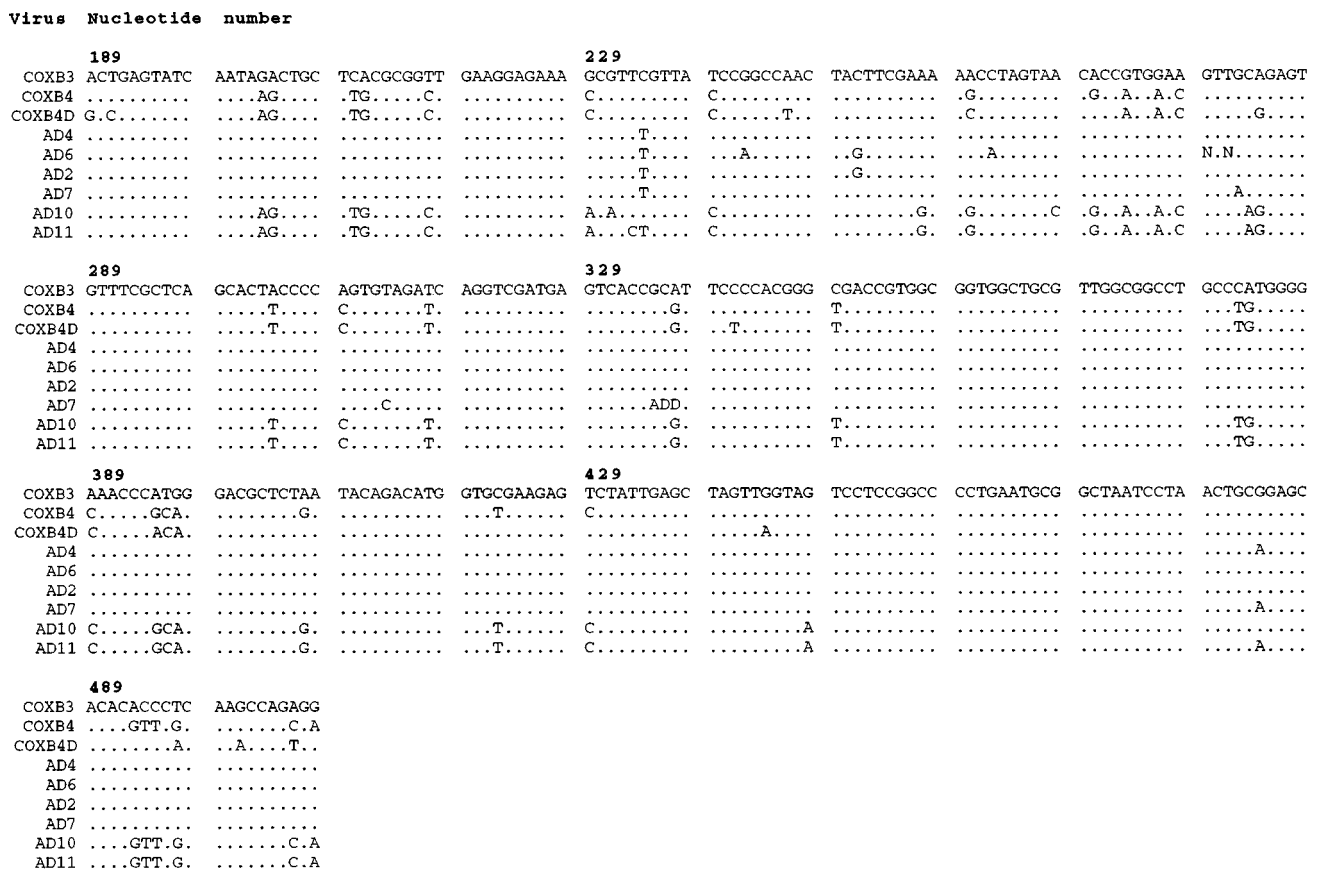

Fig. 2. Nucleotide sequence of partial 5′ nontranslated region of enteroviral isolates from cases of adult diabetes compared with standard Coxsackie virus B3 sequence. Cases of adult onset diabetes are AD4–10. The case of metabolic ketosis decompensation in the course of IDDM is AD11. Coxsackie virus B3 sequence is from GenBank. Also included are two sequences from Coxsackie B4 virus: standard B4 strain (COXB4, Genbank D00149) and human strain that is diabetogenic in mice (COXB4D). Differences are printed in full: N, mixed population of sequences containing both G and C at this position; D, deletion. Numbers correspond to nucleotide position with respect to complete genome of Coxsackie B3 (Genbank M33854).

The high frequency (42%) of enterovirus PCR-positive patients at the onset of IDDM in our series provided evidence for circulating virus at the time of diagnosis. EV sequences were found in samples collected from March 1995 to February 1996 without any relationship with epidemic outbreaks. All of these EV-positive patients were suffering from diabetic ketosis with or without acidosis, pointing to a link between EV infections and a metabolic decompensation during type I diabetes. These EV PCR results are consistent with those reported by Clements et al. [1995] in sera taken from newly diagnosed IDDM children and support evidence for the involvement of enteroviruses at the onset of IDDM either as the primary aetiological agent or as a triggering factor. However, the positive detection of EV RNA sequences in one IDDM patient from the previously diagnosed IDDM group demonstrated that circulating enteroviruses could also be detected in the course of the disease. These findings suggest the hypothesis of an IDDM phenotype associated with a

greater susceptibility to EV infections. Eberhardt et al. [1985] described that newly diagnosed IDDM patients with HLA-DR4 genotype are more likely to have recent viral infections and CVB antibodies. In the current study, three of the six IDDM patients positive for EV had an HLA-DR4 allele. Moreover, two of these six EV-positive patients did not have an HLA-DR haplotypic combination for high susceptibility to IDDM, but they were positive for HLA-DR4 genotype (Table II). In addition to HLA genes, several other EV infection susceptibility or resistance loci could be involved. The identification and the relevance of these genes to susceptibility to enteroviruses remain to be determined [Rewers and Atkinson, 1995].

The sequences detected by PCR in IDDM patients were involved in the regulation of viral translation and highly conserved among the EV group [Hellen and Wimmer, 1995]. In order to identify the serotype and the genotype of amplified EV RNA sequences, a direct DNA sequencing strategy was performed. Computer analysis of sequencing data showed that amplified EV RNA in diabetic patients was related closely to CVB3 or CVB4 and CVB4D (Fig. 2). The close similarities between CVB3 and CVB4 sequences pointed to a causal relation between genotypic determinants and the onset of IDDM (Fig. 2). However, our EV sequence data obtained from adult patients with IDDM were different from those described by Clements et al. [1995] in children with IDDM over the same genomic region. These discrepancies suggest the variability of the EV genome and the difficulty of identifying diabetogenic determinants. However, recent studies using murine models infected by pancreatopic variants of CVB4 strains allowed the identification of candidate determinants of virulence in the 5′ uncoding region and the capsid proteins VP1, VP2, and VP4 [Caggana et al., 1993; Kang et al., 1994; Ramsingh and Collins, 1995]. These findings suggest that in order to detect candidate diabetogenic determinants, further human studies in IDDM patients will need to sequence specifically these EV genomic regions. Subsequently, the identification of nucleotidic mutation or groups of mutations responsible for the induction of IDDM could be determined by functional tests in susceptible animals using recombinant CVB virus with the nucleic base(s) exchanged by site-specific mutagenesis.

In conclusion, the results of this study suggest that the pathogenesis of adult IDDM is associated in some cases with Coxsackie B virus infection. The direct role of enteroviruses in the aetiology of IDDM remains to be elucidated, and we think that the answer will come only from further prospective studies, including much larger series of IDDM patients with samples taken before and after diagnosis for viral testing.

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